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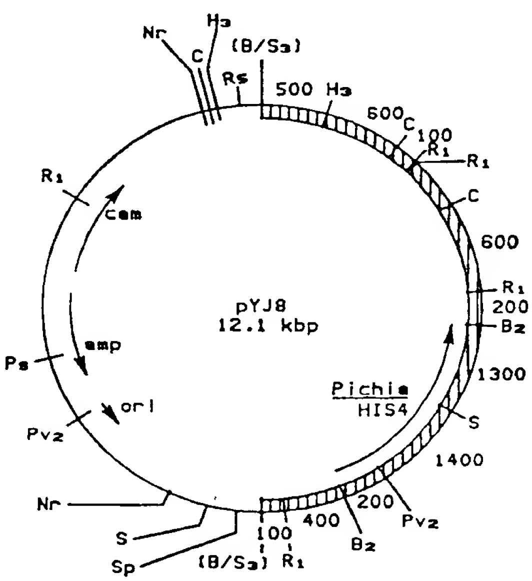
(54) Genes from pichia histidine pathway and uses thereof.

(57) Novel DNA sequences which code for the production of Pichia histidinol dehydrogenase, phosphoribosyl-ATP-cyclohydrase and phosphoribosyl-ATP-pyrophosphohydratase are provided. Novel constructs including these sequences, as well as transformed organisms therewith are provided. A method for isolation of functional genes from yeast strains of the genus Pichia is also provided. In addition, process for the integrative transformation of yeast strains of the genus Pichia is provided.



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GENES FROM *PICHIA* HISTIDINE PATHWAY AND USES THEREOF

Background

This invention relates to the field of recombinant DNA technology. In one of its aspects, the invention relates to the isolation of functional genes from yeast strains of the genus *Pichia*. In another aspect, the invention relates to DNA fragments which regulate expression of a gene product, i.e., a polypeptide. In yet another aspect, the invention relates to the integrative transformation of yeast strains of the genus *Pichia*.

Up to now, commercial efforts employing recombinant DNA technology for producing various polypeptides have centered on *Escherichia coli* as a host organism. However, in some situations *E. coli* may prove to be unsuitable as a host. For example, *E. coli* contains a number of toxic pyrogenic factors that must be eliminated from any polypeptide useful as a pharmaceutical product. The efficiency with which this purification can be achieved will, of course, vary with the particular polypeptide. In addition, the proteolytic activities of *E. coli* can seriously limit yields of some useful products. These and other considerations have led to increased interest in alternative hosts, in particular, the use of eukaryotic organisms for the production of polypeptide products is appealing.

The availability of means for the production of polypeptide products in eukaryotic systems, e.g., yeast, could provide significant advantages relative to the use of prokaryotic systems such as *E. coli* for the production of polypeptides encoded by recombinant DNA. Yeast has been employed in large scale fermentations for centuries, as compared to the relatively recent advent of large scale *E. coli* fermentations. Yeast can generally be grown to higher cell densities than bacteria and are readily adaptable to continuous fermentation processing. In fact, growth of yeast such as *Pichia pastoris* to ultra-high cell densities, i.e., cell densities in excess of 100 g/L, is disclosed by Wegner in U.S. 4,414,329 (assigned to Phillips Petroleum Co.). Additional advantages of yeast hosts include the fact that many critical functions of the organism, e.g., oxidative phosphorylation, are located within organelles, and hence not exposed to the possible deleterious effects of the organism's production of polypeptides foreign to the wild-type host cells. As a eukaryotic organism, yeast may prove capable of glycosylating expressed polypeptide products where such glycosylation is important to the bioactivity of the polypeptide product. It is also possible that as a eukaryotic organism, yeast will exhibit the same codon preferences as higher organisms, thus tending toward more efficient production of expression products from mammalian genes or from complementary DNA (cDNA) obtained by reverse transcription from, for example, mammalian mRNA.

The development of poorly characterized yeast species as host/vector systems is severely hampered by the lack of knowledge about transformation conditions and suitable vectors. In addition, auxotrophic mutations are often not available, precluding a direct selection for transformants by auxotrophic complementation. If recombinant DNA technology is to fully sustain its promise, new host/vector systems must be devised which facilitate the manipulation of DNA as well as optimize expression of

inserted DNA sequences so that the desired polypeptide products can be prepared under controlled conditions and in high yield.

A basic element employed in recombinant DNA technology is the plasmid, which is extrachromosomal, double-stranded DNA found in some microorganisms. Where plasmids have been found to naturally occur in microorganisms, they are often found to occur in multiple copies per cell. In addition to naturally occurring plasmids, a variety of man-made plasmids, or hybrid vectors, have been prepared. Included in the information encoded in plasmid DNA is that required to reproduce the plasmid in daughter cells, i.e., an autonomous replication sequence. One or more phenotypic selection characteristics must also be included in the information encoded in the plasmid DNA. The phenotypic selection characteristics permit clones of the host cell containing the plasmid of interest to be recognized and selected by preferential growth of the cells in selective media.

Objects of the Invention

An object of the invention is therefore functional genes from yeast strains of the genus *Pichia*, useful, for example, as phenotypic selection markers.

Another object of the invention is novel regulatory regions which are responsive to the presence or absence of amino acids in the culture medium.

Yet another object of the invention is the integrative transformation of yeast strains of the genus *Pichia*.

A further object of the invention is a process for producing polypeptides employing the novel DNA fragments of the invention as phenotypic selection markers and/or as regulatory regions.

These and other objects of the invention will become apparent from the disclosure and claims herein provided.

Statement of the Invention

In accordance with the present invention, I have discovered, isolated and characterized the HIS4 gene from a strain of yeast of the genus *Pichia*. In addition, I have developed a general procedure for the isolation of functional genes from yeast strains of the genus *Pichia*. The novel genes which I have isolated are useful as phenotypic selection markers in a host/vector system employing yeast strains of the genus *Pichia* as host.

In accordance with another embodiment of the invention, I have discovered, isolated and characterized a regulatory region responsive to the presence or absence of amino acids in the culture medium. This regulatory region is useful, for example, for the controlled expression of polypeptide products in yeast.

In accordance with yet another embodiment of the invention, a process for the integrative transformation of yeast of the genus *Pichia* has been developed. This method of transforming yeast provides a means for recombination of vector sequences into the chromosomal DNA of the host. This recombination results in stable maintenance of inserted DNA sequences in the host chromosomal DNA. Stable maintenance of inserted sequences is accomplished without the requirement for selective growth conditions for cell maintenance of inserted DNA sequences as extrachromosomal DNA.

Brief Description of the Figures

Figure 1 is a restriction map of plasmid YEp13.

Figure 2 is a restriction map of plasmid pYA4.

Figure 3 is a restriction map of a 6.0 kilobase pair (kbp) fragment of *Pichia* chromosomal DNA which contains

the *Pichia* HIS4 gene. Also shown are specific subfragments of interest.

Figure 4 is a restriction map of plasmid pYJ8.

Figure 5 is a restriction map of plasmid pBPf1.

Figure 6 is a restriction map of plasmid pBPf3.

The following abbreviations are used throughout this specification to represent the restriction enzymes employed:

Abbreviation	Restriction Enzyme
B	*Bam*HI
B_2	*Bgl*II
C	*Cla*I
H_3	*Hin*dIII
Nr	*Nru*I
Ps	*Pst*I
Pv_2	*Pvu*II
R_1	*Eco*RI
R_5	*Eco*RV
S	*Sal*I
Sm	*Sma*I
Sp	*Sph*I
Ss	*Sst*I
S_3	*Sau*3A
T	*Taq*I
Xb	*Xba*I
Xh	*Xho*I

In the attached figures, restriction sites employed for the manipulation of DNA fragments but which are destroyed upon ligation, are indicated by enclosing the abbreviation for the destroyed site in parenthesis. Restriction sites which have been destroyed by other means are indicated by flagging the abbreviation for the destroyed site with an asterisk.

Detailed Description of the Invention

In accordance with one embodiment of the present invention, there is provided a method for isolation of functional genes from yeast strains of the genus *Pichia*. The method is based on the recognition that *Pichia* genes and gene products have the ability to complement the defects in *Saccharomyces cerevisiae* mutant host strains, and thereby reverse the host mutant phenotypes. The method comprises:

(1) Transformation of defective *Saccharomyces cerevisiae* strains with fragments of *Pichia* chromosomal DNA, which have been recombined into *S. cerevisiae* - *E. coli* shuttle vectors;

(2) Selection of transformed strains by their ability to survive and grow in selective growth media, wherein the selective growth media are specifically devoid of the nutrients or do not provide the conditions required by the defective host strain for growth;

(3) Isolation and recovery of *Pichia* DNA fragments which contain the desired genes from the plasmids contained in the selected transformed strains.

It has been discovered that the functional genes from microorganisms of the genera *Pichia* and *Saccharomyces* are sufficiently similar to allow one to take advantage of well-characterized defective strains of *Saccharomyces cerevisiae* in order to isolate complementary functional genes from *Pichia*. For example, I have isolated genes equivalent to the *Saccharomyces* HIS3 and HIS4 genes from *Pichia*. These novel genes which I have isolated are referred to for purposes of this disclosure as the *Pichia* HIS3 and HIS4 genes. These novel genes have been isolated by transforming appropriate mutants of *S. cerevisiae* with a library of *Pichia* chromosomal DNA and selecting for transformed strains which survive in the absence of histidine supplementation in the media. Those of skill in the art recognize that one could isolate the *Pichia* LEU2 gene by transforming a *leu2* *S. cerevisiae* mutant with a library of *Pichia* chromosomal DNA and selecting for transformed strains which survive in the

absence of leucine supplementation of the media. Similarly, one could isolate the *Pichia* ARG4 gene by transforming an appropriate *S. cerevisiae* mutant with a library of *Pichia* chromosomal DNA and proceeding as above, except the selection media would be absent arginine supplementation.

Isolation and Characterization of *Pichia pastoris* HIS4 Gene

The HIS4 gene was isolated from the strain *P. pastoris* NRRL Y-11430 by partial digestion of total chromosomal DNA with *Sau*3A followed by centrifugation through sucrose gradients. (See Example II). Fragments of 5 to 20 kbp were cloned into the *Bam*HI cleavage site of the *S. cerevisiae-E. coli* shuttle vector YEp13 (ATCC 37115; Figure 1) and transformed into *E. coli*. Approximately 50,000 colonies were combined and total plasmid DNA extracted. Spheroplasts of *S. cerevisiae* strain 5799-4D (NRRL Y-15859), a *his4ABC* mutant, were mixed with about 1 μg of the YEp13 *Pichia* DNA library by the procedure of Hinnen *et al* (1978) and allowed to regenerate in a medium deficient in histidine. The transformation resulted in about $1x10^3$ prototrophic yeast colonies from a population of $5x10^7$ total regenerable spheroplasts. A parallel control sample incubated without DNA produced no colonies. Total yeast DNA was extracted from 20 of the His+ colonies and transformed back into *E. coli*. Seventeen of the yeast DNA preparations produced ampicillin resistant colonies. These cloned fragments were further characterized by restriction enzyme sizing and mapping as well as by their ability to cross hybridize with a labelled *S. cerevisiae* HIS4 fragment at low stringency (post hybridization washes in 2xSSC at 55°; SSC is 0.15 $\underline{M}$ NaCl and 15 m$\underline{M}$ sodium citrate adjusted to pH 7.0 with NaOH). Most of the plasmids contained one or more fragments which hybridized to the *S. cerevisiae* HIS4 gene. One such HIS4-containing plasmid was recloned to give a HIS4-containing plasmid designated pYJ8 and is shown in Figure 4. Plasmid pYJ8 contains pBR325 sequences, including functional

chloramphenicol and ampicillin resistance genes, as well as the *Pichia* HIS4 gene.

A detailed restriction map of the *Pichia* HIS4 gene, which comprises the enzyme activities for histidinol dehydrogenase, phosphoribosyl-ATP-cyclohydrase and phosphoribosyl-ATP-pyrophosphohydratase, is presented in Figure 3a. Referring to the 5′ end of the 6 kbp DNA fragment as the origin, the following cleavage pattern is obtained:

Restriction Enzyme	Cleavage sites	Distance from origin (bp)
R_1	4	200, 3900, 4900, 5050
Xb	2	450, 3400
B	2	700, 1100
B_2	2	750, 3500
Pv_2	2	850, 4300
S	1	2300
Xh	1	4100
C	2	4550, 5100
H_3	1	5550

By subcloning of this 6.0 kbp fragment, it was determined that a 2.7 kbp *Bgl*II fragment of this *Pichia* DNA retained the ability to transform *Pichia* or *Saccharomyces* strains defective in HIS4A, HIS4B, or HIS4C gene encoded activities. Thus, for example, *Pichia pastoris* NRRL Y-15851 (GS115), a *his*4 mutant, is able to grow on media without histidine supplementation when transformed with plasmid pBPf1, shown in Figure 5. Plasmid pBPf1 contains the 2.7 kbp *Bgl*II fragment of *Pichia* chromosomal DNA, shown in Figure 3b of the drawings.

In order to increase the utility of pBPf1, the *Bam*HI site within the 2.7 kbp *Bgl*II fragment which contains the *Pichia* HIS4 gene was destroyed prior to incorporating this fragment into pBPf1. Thus, the *Pichia* HIS4 gene, contained in a vector with no other *Bam*HI sites, was cleaved with *Bam*HI and the resulting sticky ends were filled in with a mixture of all 4 deoxynucleotides in the presence of DNA polymerase I to produce blunt ends. The newly-created blunt ends were ligated to give a modified HIS4 gene without a *Bam*HI recognition site. This modified HIS4 gene was the source of the 2.7 kbp *Bgl*II fragment used in the construction of plasmid pBPf1. The location of the destroyed *Bam*HI site

is designated by B* in Figures 5 and 6. Note that the *Bam*HI site in the polylinker of pBPf1 is a unique restriction site for the vector. In addition, the further presence of unique *Eco*RI and *Sma*I restriction sites makes pBPf1 a vector useful for exploring the ability of DNA fragments to promote and/or regulate the expression of a gene product in transformed *Pichia pastoris*.

In accordance with another embodiment of the invention, a DNA fragment comprising a regulatory region responsive to the presence or absence of amino acids in the culture medium has been isolated and characterized. Thus in Figure 3c, an approximately 600 bp *Eco*RI-*Pvu*II fragment of DNA obtained from the 5′ end of the 6.0 kbp fragment containing the *Pichia* HIS4 gene is shown. This fragment is responsive to the presence or absence of amino acids in the culture medium. In a rich medium such as YPD, the regulatory region is "off", i.e., *Pichia* HIS4 gene encoded products are no longer synthesized. Conversely, when histidine is present in low concentration in the culture medium, the regulatory region "turns on", i.e., *Pichia* HIS4 gene encoded products are synthesized.

The control of polypeptide production under the control of this novel regulatory region has been demonstrated with the novel plasmid, pBPf3, shown in Figure 6. The plasmid contains, *inter alia*, the HIS4 regulatory region and the LacZ gene. *Pichia pastoris* NRRL Y-15851 transformed with plasmid pBPf3 produces significant quantities of β-galactosidase when grown in media devoid of histidine but produces at least 10-fold less β-galactosidase when grown in a rich medium such as YPD, which includes histidine.

Integrative Transformation of *Pichia*

In accordance with yet another embodiment of the invention, a process for integrative recombination of plasmid sequences into the genome of yeast strains of the genus *Pichia* is provided. The process of integrative recombination

is referred to for purposes of this disclosure as "integrative transformation."

The transformation of *Pichia pastoris* has not been previously described. The experimental procedures for transformation of *Pichia pastoris* are presented in greater detail below (Example I). In order to develop a transformation system for *P. pastoris*, the auxotrophic mutant (GS115) NRRL Y-15851 was isolated and determined to be defective in the histidine pathway in that the strain has no detectable histidinol dehydrogenase activity.

Yeast strains of the genus *Pichia* can be transformed by enzymatic digestion of the cell walls to give spheroplasts; the spheroplasts are then mixed with the transforming DNA and incubated in the presence of calcium ions and polyethylene glycol, then regenerated in selective growth medium. The transforming DNA includes the functional gene in which the host strain is deficient, thus only transformed cells survive and grow on the selective medium employed.

Integrative transformation of *Pichia* can be accomplished by employing as the transforming DNA a vector comprising:

(i) a gene which can be selected in the *Pichia* host strain being transformed;

(ii) *Pichia* DNA sequences which have a substantial degree of homology with the genome of the *Pichia* host strain; and

(iii) additional DNA fragments selected from the group consisting of:

regulatory regions, and

polypeptide coding regions,

wherein the transforming DNA material contains essentially no autonomous replication sequence activity in *Pichia*.

Optionally, the transforming DNA can further comprise:

(iv) DNA sequences having a bacterial origin of replication and at least one marker selectible in bacteria.

The further inclusion of the DNA sequences as described in (iv) facilitates production of DNA in large quantities by allowing for propogation in bacteria. In addition, inclusion of these sequences may provide additional unique restriction sites useful for further modification of the tranforming DNA.

A suitable integrative transformation vector is represented by plasmid pYJ8, shown in Figure 4. Transformation of a host such as *Pichia pastoris* NRRL Y-15851 with plasmid pYJ8, or a derivative of pYJ8 containing additional DNA sequences, will lead to integration of the plasmid DNA into the chromosome of the host. This results from the substantial degree of homology between the defective gene of the auxotrophic mutant and the functional gene provided by the plasmid, and the purposeful absence of an ARS element in the plasmid. Unless integration occurs, a plasmid without an ARS element has no means of being passed on to daughter cells and therefore will be "lost" from future generations of host cells. The homology between the *Pichia*-derived DNA sequences of the transforming vector and the host genomic sequences provides the driving force for recombination of the vector and host DNA. As a result, plasmid DNA becomes integrated into the host chromosome.

EXAMPLES

The buffers and solutions employed in the following examples have the compositions given below:

1<u>M</u> Tris buffer	121.1 g Tris base in 800 mL of H_2O; adjust pH to the desired value by adding concentrated (35%) aqueous HCl; allow solution to cool to room temperature before final pH adjustment; dilute to a final volume of 1L.
TE buffer	1.0 m<u>M</u> EDTA in 0.01 <u>M</u> (pH 7.4) Tris buffer

LB (Luria-Bertani) Medium	5 g Bacto-tryptone 5 g Bacto-yeast extract 2.5 g NaCl in 1 L of water, adjusted to pH 7.5 with NaOH
2B Medium	0.2% NH_4PO_4 1.2% Na_2HPO_4 0.013% $MgSO_4 \cdot 7H_2O$ 0.074% $CaCl_2 \cdot 2H_2O$ 1 μg/mL thiamine 0.4% dextrose
YPD Medium	1% Bacto-yeast extract 2% Bacto-peptone 2% Dextrose
SD Medium	6.75 g yeast nitrogen base without amino acids (DIFCO) 2% Dextrose in 1 L of water
HAM	0.67% yeast nitrogen base without amino acids (DIFCO) 1% dextrose 2% histidine assay medium (DIFCO) 50 μg/mL each of: isoleucine leucine lysine methionine glutamine
SED	1 <u>M</u> Sorbitol 25 m<u>M</u> EDTA 50 m<u>M</u> DTT

SCE Buffer	9.1 g Sorbitol
	1.47 g Sodium citrate
	0.168 g EDTA
	50 mL H_2O
	--pH to 5.8 with HCl
CaS	1 M Sorbitol
	10 mM $CaCl_2$
	--filter sterilize
PEG Solution	20% polyethylene glycol-3350
	10mM $CaCl_2$
	10mM Tris-HCl (pH 7.4)
	--filter sterilize
SOS	1 M Sorbitol
	0.3x YPD medium
	10 mM $CaCl_2$

The following abbreviations are used throughout the examples with the following meaning:

EDTA	ethylenediamine tetraacetic acid
SDS	sodium dodecyl sulfate
DTT	dithiothreitol

EXAMPLE I

Pichia pastoris Transformation Procedure

A. Cell Growth

1. Inoculate a colony of *Pichia pastoris* GS115 (NRRL Y-15851) into about 10 mL of YPD medium and shake culture at 30°C for 12-20 hrs.

2. After about 12-20 hrs., dilute cells to an OD_{600} of about 0.01-0.1 and maintain cells in log growth phase in YPD medium at 30°C for about 6-8 hrs.

3. After about 6-8 hrs, inoculate 100 mL of YPD medium with 0.5 mL of the seed culture at an OD_{600} of about 0.1 (or equivalent amount). Shake at 30°C for abour 12-20 hrs.

4. Harvest culture when OD_{600} is about 0.2-0.3 (after approximately 16-20 hrs) by centrifugation at 1500 g for 5 minutes.

B. <u>Preparation of Spheroplasts</u>

1. Wash cells once in 10 mL of sterile water. (All centrifugations for steps 1-5 are at 1500 g for 5 minutes.)

2. Wash cells once in 10 mL of freshly prepared SED.

3. Wash cells twice in 10 mL of sterile 1 <u>M</u> Sorbitol.

4. Resuspend cells in 10 mL SCE buffer.

5. Add 5-10 µL of 4 mg/mL Zymolyase 60,000 (Miles Laboratories). Incubate cells at 30°C for about 30-60 minutes.

Since the preparation of spheroplasts is a critical step in the transformation procedure, one should monitor spheroplast formation as follows: add 100 µL aliquots of cells to 900 µL of 5% SDS and 900 µL of 1 <u>M</u> Sorbitol before or just after the addition of zymolyase and at various times during the incubation period. Stop the incubation at the point where cells lyse in SDS but not in sorbitol (usually between 30 and 60 minutes of incubation).

6. Wash spheroplasts twice in 10 mL of sterile 1 <u>M</u> Sorbitol by centrifugation at 1000 g for 5-10 minutes. (The time and speed for centrifugation may vary; centrifuge enough to pellet spheroplasts but not so much that they rupture from the force.)

7. Wash cells once in 10 mL of sterile CaS.

8. Resuspend cells in total of 0.6 mL of CaS.

C. <u>Transformation</u>

1. Add DNA samples (up to 20 µL volume) to 12 X 75 mm sterile polypropylene tubes. (DNA should be in water or TE buffer; for maximum transformation frequencies with small

amounts of DNA, it is advisable to add about 1 μL of 5 mg/mL sonicated *E. coli* DNA to each sample.)

2. Add 100 μL of spheroplasts to each DNA sample and incubate at room temperature for about 20 minutes.

3. Add 1 mL of PEG solution to each sample and incubate at room temperature for about 15 minutes.

4. Centrifuge samples at 1000 g for 5-10 minutes and decant PEG solution.

5. Resuspend samples in 150 μL of SOS and incubate for 30 minutes at room temperature.

6. Add 850 μL of sterile 1 <u>M</u> Sorbitol and plate aliquots of samples as described below.

D. <u>Regeneration of Spheroplasts</u>

1. Recipe for Regeneration Agar Medium:

a. Agar-Sorbitol- 9 g Bacto-agar, 54.6 g Sorbitol, 240 mL H_2O, autoclave.

b. 10X Glucose- 20 g Dextrose, 100 mL H_2O, autoclave.

c. 10X SC- 6.75 g Yeast Nitrogen Base without amino acids, 100 mL H_2O, autoclave. (Add any desired amino acid or nucleic acid up to a concentration of 200 μg/mL before or after autoclaving.)

d. Add 30 mL of 10X Glucose and 30 mL of 10X SC to 300 mL of the melted Agar-Sorbitol solution. Add 0.6 mL of 0.2 mg/mL biotin and any other desired amino acid or nucleic acid to a concentration of 20 μg/mL. Hold melted Regeneration Agar at 55-60°C.

2. Plating of Transformation Samples:

Pour bottom agar layer of 10 mL Regeneration Agar per plate at least 30 minutes before transformation samples are ready. Distribute 10 mL aliquots of Regeneration Agar to tubes in a 45-50°C bath during the period that transformation samples are in SOS. Add aliquots of transformation samples described above to tubes with Regeneration Agar and pour onto bottom agar layer of plates. Add a quantity of each sample to 10 mL aliquots of melted Regeneration Agar held at 45-50°C

and pour each onto plates containing a solid 10 mL bottom agar layer of Regenation Agar.

3. Determination of Quality of Spheroplast Preparation: Remove 10 μL of one sample and dilute 100 times by addition to 990 μL of 1 M Sorbitol. Remove 10 μL of the 100 fold dilution and dilute an additional 100 times by addition to a second 990 μL aliquot of 1 M Sorbitol. Spread plate 100 μL of both dilutions on YPD agar medium to determine the concentration of unspheroplasted whole cells remaining in the preparation. Add 100 μL of each dilution to 10 mL of Regeneration Agar supplemented with 40 μg/mL histidine to determine total regeneratable spheroplasts. Good values for a transformation experiment are 1-3 X 10^7 total regeneratable spheroplasts/mL and about 1 X 10^3 whole cells/mL.

4. Incubate plates at 30° C for 3-5 days.

Example II

Isolation Of *Pichia Pastoris* HIS4 Gene

A. Strains

The strains employed include:

(a) *Pichia pastoris* strain NRRL Y-11430;

(b) *Pichia pastoris* strain NRRL Y-15851 (GS115-*his4*);

(c) *S. cerevisiae* strain 5799-4D (a *his*4-260 *his*4-39; NRRL Y-15859); and

(d) *E. coli* strain 848 (F^- *met thi gal* T_1^R $\phi 80^S$ *hsd*R^- *hsd*M^+).

B. Plasmids

pYA2, which consists of the *S. cerevisiae* HIS4 gene on a 9.3 kbp *Pst*I fragment inserted at the *Pst*I site of pBR325 was the source of the *S. cerevisiae* HIS4 gene fragments and has been deposited in an *E. coli* host and is available to the public as NRRL B-15874.

YEp13 is available from the American Type Culture Collection and has been assigned accession number ATCC 37115.

C. Media

Pichia pastoris was grown in YPD (rich) or IMG (minimal) media. IMG, a minimal medium, consists of the following:

1. IM_1 Salts at a final concentration of 36.7 mM KH_2PO_4, 22.7 mM $(NH_4)_2SO_4$, 2.0 mM $MgSO_4 \cdot 7H_2O$, 6.7 mM KCl, 0.7 mM $CaCl_2 \cdot 2H_2O$, prepared as a 10x stock solution and autoclaved;

2. Trace Salts at a final concentration of 0.2 μM $CuSO_4 \cdot 5H_2O$, 1.25 μM KI, 4.5 μM $MnSO_4 \cdot H_2O$, 2.0 μM $NaMoO_4 \cdot 2H_2O$, 0.75 μM H_3BO_3, 17.5 μM $ZnSO_4 \cdot 7H_2O$, 44.5 μM $FeCl_3 \cdot 6H_2O$, prepared as a 400x stock solution and filter sterilized;

3. 0.4 μg/mL biotin; and

4. 2% dextrose.

E. coli was cultured in either LB medium or 2B medium supplemented with 100 μg/mL tryptophan, and 0.2% Casamino acids.

D. DNA Isolation

1. Large Scale Preparations of Yeast DNA.

Both *Pichia pastoris* and *S. cerevisiae* DNA preparations were carried out by growing yeast cells in 100 mL of minimal medium until A_{600} equals 1-2 and then harvesting the cells by centrifugation at 2,000 g for 5 minutes. The cells were washed once in H_2O, once in SED, once in 1 M sorbitol and then suspended in 5 mL of 0.1 M Tris-HCl (pH 7.0) which is 1 M in sorbitol. The cells were mixed with 50-100 μL of a 4 mg/mL solution of Zymolase 60,000 (Miles Laboratories) and incubated at 30°C for 1 hour to digest the cell walls. The spheroplast preparation was then centrifuged at 1000 g for 5-10 minutes and suspended in Lysis buffer (0.1% SDS, 10 mM Tris-HCl, (pH 7.4), 5 mM EDTA and 50 mM NaCl). Proteinase K (Boehringer Mannheim) and RNase A (Sigma) were each added to 100 μg/mL and the mixture incubated at 37°C for 30 minutes. DNA was deproteinized by gently mixing the preparation with an equal volume of

chloroform containing isoamyl alcohol (24:1, v/v) and the phases were separated by centrifugation at 12,000 g for 20 minutes. The upper (aqueous) phase was drawn off into a fresh tube and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The phases were separated as before and the top phase placed in a tube containing 2-3 volumes of cold 100% ethanol. The sample was gently mixed and DNA was collected by spooling onto a plastic rod. The DNA was immediately dissolved in 1 mL of TE buffer and dialyzed overnight at 4°C against 100 volumes TE buffer.

2. <u>Small Scale Yeast DNA Preparations</u>.

Five mL of yeast cultures in minimal medium were grown until A_{500} equals 1-5 and harvested by centrifugation at 2,000 g for 5 minutes. Cells were suspended in 1 mL of SED and transferred to a 1.5 mL microfuge tube, washed once in 1 <u>M</u> sorbitol and resuspended in 0.5 mL of 0.1 M Tris-HCl (pH 7.4) which is 1 <u>M</u> sorbitol. Zymolyase 60,000 (Miles Laboratories; 10 µL of a 4 mg/mL solution) was added to each sample and the cells were incubated for 30-60 minutes at 30°C. Cells were then centrifuged for 1 minute, suspended in the Lysis buffer and incubated at 65-70°C. After 15 minutes the samples were mixed with 100 µL of 5 <u>M</u> potassium acetate, held in an ice bath for 15 minutes and centrifuged for 5 minutes. The supernatants were decanted into a fresh microfuge tube containing 1 mL of 100% ethanol, mixed and immediately centrifuged for 10 seconds. Finally, the DNA pellets were air dried for 10-15 minutes and dissolved in 50 µL of TE buffer.

3. <u>Large Scale *E. coli* DNA Preparations</u>.

E. coli cultures for large scale (0.5-1 L) plasmid preparations were grown at 37°C with shaking in 2B medium supplemented as described above and with the appropriate antibiotic. For cells which contained pBR322 derived

plasmids, cultures were grown to an A_{550} of about 0.7 at which time sufficient chloramphenicol was added to give a concentration of 100 μg/mL and cells harvested approximately 15 hours later. Strains which contained pBR325 derived plasmids were inoculated into the supplemented 2B medium at a starting A_{550} of about 0.01-0.05 and incubated with shaking at 37°C for 20-24 hours before harvesting. Plasmids were isolated by the alkaline lysis method described by Birnboim and Doly (1979).

4. Small Scale *E. coli* DNA Preparations.

For small scale rapid plasmid isolations, 2 mL cultures in the supplemented 2B medium with antibiotic were grown overnight at 37°C with shaking and harvested by centrifugation in 1.5 mL microfuge tubes. Plasmids were isolated by the alkaline lysis method described by Birnboim and Doly (1979).

E. Restriction of DNA and Fragment Isolation

Restriction enzymes were obtained from New England Biolabs and Bethesda Research Laboratories and digestions were performed by routine techniques. Restriction mappings were carried out by comparing parallel digestions of plasmids with and without insert DNA. Restriction fragments were purified by electroelution from agarose gels into Whatman 3 MM paper strips backed by dialysis tubing. The fragments were recovered from the paper and tubing by 3-4 washings with 0.1-0.2 mL volumes of a solution which contained 0.1 M NaCl, 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Finally, the fragments were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and redissolved in a small volume of TE buffer.

F. Construction of *Pichia pastoris* Library in *E. Coli*

For the *Pichia pastoris* DNA-YEp13 library construction, 100 μg of YEp13 was digested to completion with

*Bam*HI and treated with calf intestinal alkaline phosphatase to remove the terminal 5′ phosphate from the DNA. A 100 μg aliquot of wild type *Pichia pastoris* DNA from *Pichia pastoris* NRRL Y-11430) was partially digested with 10 units of *Sau*3A I by incubation for 5 minutes at 37°C in a total volume of 1 mL. Fragments of 5 to 20 kbp were size selected by centrifugation through 5-20% sucrose gradients. One μg of the vector and 2 μg of the *Pichia Sau*3AI fragments were mixed with 20 units of T4 DNA ligase (Bethesda Research Laboratories) in a total volume of 200 μL and incubated overnight at 4°C. The ligated DNAs were transformed into *E. coli* by adding the entire ligation reaction mix to 2 mL of competent *E. coli* 848 cells and incubating for 15 minutes at 0°C. The mixture was warmed to 37°C for 5 minutes after which time 40 mL of LB medium was added and the 37°C incubation continued for an additional 1 hour. Ampicillin was then added to give a total concentration of 100 μg/mL and the incubation continued for a second hour. Finally, the cells were centrifuged for 10 minutes at 3,000 g, resuspended in 1 mL of fresh LB medium and spread in equal aliquots on 10 LB agar plates containing 100 μg/mL of ampicillin. The approximately 50,000 colonies which resulted were scraped from the plates and a portion of the cells was inoculated into 500 mL of the supplemented 2B medium at a starting A_{550} of about 0.1. The culture was grown and plasmid was extracted as described above. Of the colonies which were pooled for the library, 96 out of 100 tested were tetracycline sensitive and 7 out of 10 examined contained plasmids with insert DNA.

G. Southern Hybridizations

Hybridizations were carried out by the method of Southern (1975). For transfer of large or supercoiled DNA molecules to nitrocellulose, DNA was first partially hydrolyzed by soaking agarose gels in 0.25 M HCl for 10 minutes prior to alkali denaturation. All hybridizations of

labelled fragments were performed in the presence of 50% formamide, 6x SSC, 5x Denhardt's, 0.1% SDS, 1 mM EDTA, and 100 μg/mL denatured herring sperm DNA at 42°C. Post-hybridization washes for hybridization of labelled fragments from the *S. cerevisiae* HIS4 gene to *Pichia pastoris* DNA were carried out under the low stringency conditions of 2x SSC, 1 mM EDTA, 0.1% SDS and 1.0% sodium pyrophosphate at 55°C.

H. ^{32}P-Labelling

Nick translations were carried out according to the procedure of Rigby et al (1977).

I. Yeast Transformations

S. cerevisiae transformations were carried out by the spheroplast generation method of Hinnen *et al* (1978).

Pichia pastoris transformations were performed following the procedure described above.

J. Isolation of *Pichia* HIS4 Gene

DNA fragments which contained the *Pichia* HIS4 gene were isolated from a *Pichia* DNA library by their ability to complement *S. cerevisiae his4* strains. The library was composed of 5-20 kb *Sau*3AI partial digestion fragments of wild type *Pichia* DNA inserted into the *Bam*HI site of the *S. cerevisiae-E. coli* shuttle vector YEp13. Spheroplasts of *S. cerevisiae* NRRL Y-15859 (5799-4D; a *his*4ABC$^-$ strain) were generated by the technique of Hinnen *et al* (1978), mixed with the *Pichia* DNA library and allowed to regenerate in a medium deficient in histidine. The transformation resulted in about 1×10^3 prototrophic yeast colonies from a population of 5×10^7 total regeneratable spheroplasts. Total yeast DNA was extracted from 20 of the His+ colonies and transformed into *E. coli*. Seventeen of the yeast DNA preparations produced ampicillin resistant colonies and each contained plasmid comprised of YEp13 plus insert DNA. To confirm that the His+ transforming plasmids contained the *Pichia* HIS4 gene and not

a DNA fragment with suppressor activity, restriction digests of the plasmids were hybridized to a labelled DNA fragment containing a large portion of the *S. cerevisiae* HIS4 gene and washed at low stringency. Each of the plasmids which complemented the *his*4 *S. cerevisiae* strains contained sequences which hybridized to the *S. cerevisiae* HIS4 gene.

One of the His^+ transforming plasmids was selected for further characterization. A detailed restriction map of the 6.0 kbp fragment is presented in Figure 3. The smallest subfragment capable of transforming the *his*4 mutant NRRL Y-15851 (GS115) at high frequency was determined to be 2.7 kbp *Bgl*II fragment (see Figure 3) by transforming GS115 with portions of the 6.0 kbp fragment. This *Bgl*II fragment was also capable of transforming the *S. cerevisiae his4* mutant NRRL Y-15859 at high frequency. Thus the HIS4 gene isolated and characterized in accordance with the invention is believed to contain the HIS4A, HIS4B and HIS4C gene functions which have been identified in *S. cerevisiae*, i.e., phosphoribosyl-ATP-cyclohydrase, phosphoribosyl-ATP-pyrophosphohydratase and histidinol dehydrogenase, respectively.

EXAMPLE III

Construction Of Regulatory Region-LacZ Gene Fusions

In order to identify a DNA fragment which contains the *Pichia* HIS4 promoter and regulatory sequences, plasmid pYJ8 was restricted with *Eco*RI and *Pvu*II and electroeluted. A 600 bp fragment of *Pichia* DNA was collected and ligated into plasmid pBPf1 which had been cleaved at the polylinker site with *Eco*RI and *Sma*I. The resulting plasmid, pBPf3, was used to transform *Pichia pastoris* NRRL Y-15851, which was then used in further studies directed to the production of β-galactosidase under the control of the *Pichia* HIS4 regulatory region.

EXAMPLE IV

Expression of β-Galactosidase Under Amino Acid Control

A. Plate Culture Assays

P. pastoris NRRL Y-15851 transformed with pBPf3 was streaked onto YPD and SD medium 2% agar plates supplemented with 0.4 μg/mL of biotin and 40 μg/mL of Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The SD-containing plates were adjusted to a pH of 7 by addition of monobasic potassium phosphate. The principle of the Xgal assay is that β-galactosidase, if present in the cells, will react with Xgal to produce a compound with a blue color. The resulting blue color can be visually detected. Blue color developed in the pBPf3 transformed *P. pastoris* cells within about 4-5 hours on the minimal (SD medium) plate, while about 1 week incubation was required for blue color development to occur on plates containing the rich (YPD) medium. As controls, neither untransformed *P. pastoris* cells, nor pBPf1 transformed *P. pastoris* cells nor pBPf2 (a derivative of pBPf1 with a 400 bp R_1-B_2 fragment from the 5′ terminus of the *Pichia* HIS4 gene) transformed *P. pastoris* cells turned blue under any conditions tested. These observations suggest that the 600 bp R_1-R_2 *Pichia* HIS4 fragment-LacZ gene fusion is expressed and is regulated under an amino acid control system.

B. Liquid Culture Assays

P. pastoris NRRL Y-15851 was transformed with plasmid pBPf3. Transformed cells were inoculated into SD medium supplemented with 0.4 μg/mL of biotin and grown at 30°C with shaking until the A_{600} per mL of solution was about 0.7. Then 3.5 mL aliquots of culture were added to each of 2 flasks containing 96.5 mL of one of the following media:

I. SD medium plus 0.4 μg/mL biotin; and

II. HAM.

These 2 cultures were grown logarithmically at 30°C with shaking for 80 hours. Samples were removed after specific time periods and assayed for β-galactosidase.

β-Galactosidase Assay

1. Solutions required:

Z-buffer:		Final concentration
$Na_2HPO_4 \bullet 7H_2O$	16.1 g	0.06 M
NaH_2PO_4	5.5 g	0.04 M
KCl	0.75 g	0.01 M
$MgSO_4 \bullet 7H_2O$	0.246 g	0.001 M
2-mercaptoethanol	2.7 mL	0.05 M

fill up to 1L; pH should be 7

O-Nitrophenyl-β-D-galactoside (ONPG):

Dissolve 400 mg ONPG (Sigma N-1127) in 100 mL of distilled water to make a 4 mg/mL ONPG solution.

2. Assay Procedure:

i. Withdraw an aliquot from the culture medium (0.1-0.5 OD_{600} of yeast cells), centrifuge and wash cell pellet with water.

ii. Add 1 mL of Z buffer to the cell pellet, 30 μL of $CHCl_3$ and 30 μL of 0.1% SDS, vortex, incubate 5 minutes at 30°C.

iii. Start reaction by adding 0.2 mL of ONPG (4 mg/mL), vortex.

iv. Stop reaction by adding 0.5 mL of a 1 M Na_2CO_3 solution at appropriate time points A_{420}<1).

v. Read absorbance of supernatant at 420 nm.

3. Calculation of β-galactosidase Units:

1 U = 1 nmole of orthonitrophenol (ONP) formed per minute at 30°C and a pH 7.

1 nmole of ONP has an absorbance at 420 nm (A_{420}) of 0.0045 with a 1 cm pathlength; thus, an absorbance of 1 at 420 nm represents 222 nmole ONP/mL, or 378 nmole/1.7 mL since the total volume of supernatant being analyzed is 1.7 mL. Hence, Units expressed in the Tables are calculated:

$$U = \frac{A_{420}}{t(\text{min})} \times 378$$

Results of these experiments are summarized in the Table.

Table

Medium	β-galactosidase activity (u/A_{600}), hrs. 24	45	72
SD	63	113	173
HAM	97	280	225

The results presented in the Table indicate that β-galactosidase is expressed under control of the *Pichia* HIS4 promoter region.

EXAMPLE V

Integrative Transformation of *Pichia*

To demonstrate integrative recombination of a vector into the genome of *P. pastoris*, *P. pastoris* NRRL Y-15851 was transformed with plasmid pYJ8 (see Figure 4), a vector which can be selected in *his4* *Pichia* hosts such as NRRL Y-15851. Plasmid pYJ8 has approximately 6.0 kbp of DNA sequences which are homologous to the *Pichia* genome. Further, plasmid pYJ8 has no detectable autonomous

replication sequence activity in *Pichia* hosts. The transformation of NRRL Y-15851 with pYJ8 resulted in the formation of about 50 His^+ colonies per μg of pYJ8. The presence of pYJ8 sequences in the *P. pastoris* genome was demonstrated as follows: Transformant colonies were picked from the regeneration agar plate and streaked onto an SD medium agar plate. The SD plate was incubated at 30°C for 3 days after which time a single colony of each transformant was inoculated into a flask of IMG medium. The IMG cultures were grown at 30°C with shaking to an A_{600} of about 1-2 and then harvested by centrifugation. DNA from the yeast cultures was extracted as described above, and 1-2 μg of each DNA (unrestricted) was electrophoresed at 30 Volts and 30 mAmps for 10-15 hours into 0.8% agarose gels, transferred to nitrocellulose and hybridized to ^{32}P-labelled pBR322 as described above. As controls, a sample containing 10 ng of plasmid pYJ8 isolated from *E. coli* and a sample containing 1-2 μg of untransformed *Pichia pastoris* GS115 DNA were electrophoresed in parallel with the experimental samples. In each of the pYJ8-*Pichia* transformants examined, the labelled pBR322 probe hybridized to the high molecular weight *Pichia* chromosomal DNA. In the control, no labelled probe hybridized to the untransformed *Pichia* DNA.

The examples have been provided merely to illustrate the practice of my invention and should not be read so as to limit the scope of my invention or the appended claims in any way. Reasonable variation and modification, not departing from the essence and spirit of my invention, are contemplated to be within the scope of patent protection desired and sought.

Bibliography

Birnboim and Doly (1979) Nucl. Acids Res. 7, 1513-1523.

Hinnen *et al* (1978) Proc. Nat. Acad. Sci., USA 75, 1929-1933.

Rigby *et al* (1977) J. Mol. Biol. 113, 237.

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The following part of the description are preferred embodiments 1 to 30 presented in the format of claims.

1. A method for the isolation of functional genes from yeast strains of the genus *Pichia* comprising:

(a) preparing a library by treating *Pichia* total DNA with an appropriate restriction enzyme to give *Pichia* DNA fragments and cloning said DNA fragments into a *Saccharomyces cerevisiae* - *Escherichia coli* shuttle vector;

(b) transforming with said library a mutant *Saccharomyces cerevisiae* host strain which has a selectable phenotype as a result of a defect in at least one gene product;

(c) growing the transformed strains under selective growth conditions; wherein said selective growth conditions do not provide the conditions required by the defective host strain for growth;

(d) isolating plasmid DNA from said transformed strains; and

(e) recovering *Pichia* DNA inserts from said plasmids.

2. A method in accordance with claim 1 wherein said *Saccharomyces cerevisiae* - *Escherichia coli* shuttle vector is YEp13.

3. A method in accordance with claim 1 wherein said host *Saccharomyces cerevisiae* strain is defective in an amino acid biosynthetic pathway.

4. A method in accordance with claim 3 wherein said host *Saccharomyces cerevisiae* strain is defective in the HIS4 gene.

5. A method in accordance with claim 4 wherein said host strain is *Saccharomyces cerevisiae* NRRL Y-15859.

6. A method in accordance with claim 1 wherein said selective growth conditions comprise yeast minimal media absent histidine.

7. A gene coding for the production of histidinol dehydrogenase, or a sub-unit thereof or an equivalent of such gene or sub-unit.

8. A gene in accordance with claim 7 further coding for the production of phosphoribosyl-ATP-cyclohydrase and phosphoribosyl-ATP-pyrophosphohydratase or a sub-unit thereof or an equivalent of such a gene or sub-unit.

9. A gene in accordance with claim 7 wherein said gene is characterized by the restriction map in FIG. 3b of the drawings.

10. A gene in accordance with claim 7 further comprising flanking regions of chromosomal DNA as characterized by the restriction map in FIG. 3a of the drawings.

11. A DNA fragment comprising a regulatory region; wherein said regulatory region is responsive to the presence or absence of nutrients in the culture medium with which a host organism for said DNA fragment is in contact and wherein said regulatory region is capable of controlling the production of polypeptides when positioned at the 5′ end of the DNA which codes for the production of said polypeptides, and mutants of said DNA fragment.

12. A DNA fragment in accordance with claim 11 wherein said fragment is characterized by the restriction map in FIG. 3c of the drawings.

13. A DNA fragment in accordance with claim 11 further comprising the LacZ gene positioned at the 3′ end of said regulatory region.

14. A DNA fragment in accordance with claim 13 wherein said fragment is characterized by the 4.2 kbp *Eco*RI-*Nru*I fragment of the restriction map in FIG. 6 of the drawings.

15. Hybrid plasmid pYJ8 having the restriction map shown in FIG. 4 of the drawings.

16. Hybrid plasmid pBPf3 having the restriction map shown in FIG. 6 of the drawings.

17. *Escherichia coli* NRRL B-15889 (LE392-pYJ8).

18. *Escherichia coli* NRRL B-15892 (LE392-pBPf1).

19. A process comprising:

(a) culturing *Escherichia coli* NRRL B-15889 (LE392-pYJ8) in a nutrient medium;

(b) disrupting the cultured cells; and

(c) recovering plasmid pYJ8 from the disrupted cells.

20. A process in accordance with claim 19 further comprising:

(d) digesting plasmid pYJ8 with one of the restriction enzyme combinations selected from the group consisting of;

*Nru*I,
*Eco*RV and *Nru*I,
*Eco*RV and *Sph*I, and
*Nru*I and *Sph*I; and

(e) recovering a DNA fragment of about 6.0 kbp.

21. A process in accordance with claim 19 further comprising:

(d) digesting plasmid pYJ8 with the restriction enzyme *Bgl*II; and

(e) recovering a DNA fragment of about 2.7 kbp.

22. A process in accordance with claim 19 further comprising:

(d) digesting plasmid pYJ8 with the restriction enzymes *Eco*RI and *Pvu*II; and

(e) recovering a DNA fragment of about 600 base pairs.

23. A process for the integrative transformation of yeasts of the genus *Pichia* comprising transforming host strains of the genus *Pichia* with recombinant DNA material; wherein said recombinant DNA material comprises:

(i) a gene which can be selected in a *Pichia* host strain;

(ii) *Pichia* DNA sequences which have a substantial degree of homology with the genome of the host *Pichia* strain; and

(iii) additional DNA selected from the group consisting of:

regulatory regions, and

polypeptide coding regions;

wherein said recombinant DNA material contains essentially no autonomous replication activity in *Pichia*.

24. A process in accordance with claim 23 wherein said recombinant DNA material further comprises:

(iv) DNA sequences having a bacterial origin of replication and at least one marker selectible in bacteria.

25. A process in accordance with claim 23 wherein said recombinant DNA material comprises plasmid pYJ8.

26. A process in accordance with claim 23 wherein said recombinant DNA material comprises plasmid pYJ8Δ*Cla*.

27. A process for controlling the expression of polypeptide in a transformed yeast strain comprising culturing said transformed yeast strain in the presence or absence of added nutrients; wherein said transformed yeast strain has been transformed with recombinant DNA material; wherein said recombinant DNA material comprises a regulatory region and a polypeptide coding region; wherein said regulatory region is responsive to the presence of nutrients in the culture medium with which the transformed yeast strain is in contact, wherein said regulatory region is capable of controlling the production of polypeptides when positioned at the 5′ end of the DNA coding region which codes for the production of said polypeptide; wherein said polypeptide coding region is expressed in the absence of added nutrient and not expressed in the presence of added nutrient.

28. A process in accordance with claim 27 wherein said transformed yeast strain is a yeast of the genus *Pichia*.

29. A process in accordance with claim 27 wherein said recombinant DNA material is plasmid pBPf3.

30. A process in accordance with claim 27 wherein said added nutrient comprises histidine.

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DATUM October 29, 1985
31 745-EP
D/la

Claims

1. A method for the isolation of functional genes from yeast strains of the genus Pichia comprising:
   (a) preparing a library by treating Pichia total DNA with an appropriate restriction enzyme to give Pichia DNA fragments and cloning said DNA fragments into a Saccharomyces cerevisiae - Escherichia coli shuttle vector;
   (b) transforming with said library a mutant Saccharomyces cerevisiae host strain which has a selectable phenotype as a result of a defect in at least one gene product;
   (c) growing the transformed strains under selective growth conditions; wherein said selective growth conditions do not provide the conditions required by the defective host strain for growth;
   (d) isolating plasmid DNA from said transformed strains; and
   (e) recovering Pichia DNA inserts from said plasmids.

2. The method of claim 1 characterized in that said Saccharomyces cerevisiae - Escherichia coli shuttle vector is YEp13; in particular wherein said host Saccharomyces cerevisiae strain is defective in an amino acid biosynthetic pathway; in particular wherein said host Saccharomyces cerevisiae

strain is defective in the HIS4 gene; in particular wherein said host strain is Saccharomyces cerevisiae NRRL Y-15859; in particular wherein said selective growth conditions comprise yeast minimal media absent histidine.

3. A gene coding for the production of histidinol dehydrogenase, or a sub-unit thereof or an equivalent of such gene or sub-unit; in particular further coding for the production of phosphoribosyl-ATP-cyclohydrase and phosphoribosyl-ATP-pyrophosphohydratase or a sub-unit thereof or an equivalent of such a gene or sub-unit; in particular wherein said gene is characterized by the restriction map in Fig. 3b of the drawings; in particular further comprising flanking regions of chromosomal DNA as characterized by the restriction map in Fig. 3a of the drawings.

4. A DNA fragment comprising a regulatory region; wherein said regulatory region is responsive to the presence or absence of nutrients in the culture medium with which a host organism for said DNA fragment is in contact and wherein said regulatory region is capable of controlling the production of polypeptides when positioned at the 5' end of the DNA which codes for the production of said polypeptides, and mutants of said DNA fragment; in particular wherein said fragment is characterized by the restriction map in Fig. 3c of the drawings; in particular further comprising the LacZ gene positioned at the 3' end of said regulatory region; in particular wherein said fragment is characterized by the 4.2 kbp EcoRI-NruI fragment of the restriction map in Fig. 6 of the drawings.

5. Hybrid plasmid pYJ8 having the restriction map shown in Fig. 4 of the drawings or hybrid plasmid pBPf3 having the restriction map shown in Fig. 6 of the drawings.

6. Escherichia coli NRRL B-15889 (LE392-pYJ8) or Escherichia

coli NRRL B-15892 (LE392-pBPf1).

7. A process comprising:

(a) culturing Escherichia coli NRRL B-15889 (LE392-pYJ8) in a nutrient medium;

(b) disrupting the cultured cells; and

(c) recovering plasmid pYJ8 from the disrupted cells; in particular further comprising:

(d) digesting plasmid pYJ8 with one of the restriction enzyme combinations selected from the group consisting of

NruI,
EcoRV and NruI,
EcoRV and SphI, and
NruI and SphI; and

(e) recovering a DNA fragment of about 6.0 kbp; in particular further comprising:

(d') digesting plasmid pYJ8 with the restriction enzyme BglII; and

(e') recovering a DNA fragment of about 2.7 kbp; in particular further comprising:

(d'') digesting plasmid pYJ8 with the restriction enzymes EcoRI and PvuII; and

(e'') recovering a DNA fragment of about 600 base pairs.

8. A process for the integrative transformation of yeasts of the genus Pichia comprising transforming host strains of the genus Pichia with recombinant DNA material; wherein said recombinant DNA material comprises:

(i) a gene which can be selected in a Pichia host strain;

(ii) Pichia DNA sequences which have a substantial degree of homology with the genome of the host Pichia strain; and

(iii) additional DNA selected from the group consisting of:

regulatory regions, and
polypeptide coding regions;

wherein said recombinant DNA material contains essentially no autonomous replication activity in

Pichia; in particular wherein said recombinant DNA material further comprises:

(iv) DNA sequences having a bacterial origin of replication and at least one marker selectible in bacteria;

in particular wherein said recombinant DNA material comprises plasmid pYJ8; in particular wherein said recombinant DNA material comprises plasmid pYJ8ΔCla.

9. A process for controlling the expression of polypeptide in a transformed yeast strain comprising culturing said transformed yeast strain in the presence or absence of added nutrients; wherein said transformed yeast strain has been transformed with recombinant DNA material; wherein said recombinant DNA material comprises a regulatory region and a polypeptide coding region; wherein said regulatory region is responsive to the presence of nutrients in the culture medium with which the transformed yeast strain is in contact, wherein said regulatory region is capable of controlling the production of polypeptides when positioned at the 5' end of the DNA region which codes for the production of said polypeptide; wherein said polypeptide coding region is expressed in the absence of added nutrient and not expressed in the presence of added nutrient.

10. The process of claim 9 characterized in that said transformed yeast strain is a yeast of the genus Pichia; in particular wherein said recombinant DNA material is plasmid pBPf3; in particular wherein said added nutrient comprises histidine.

European Patent Office

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Application number:

85113734.9

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

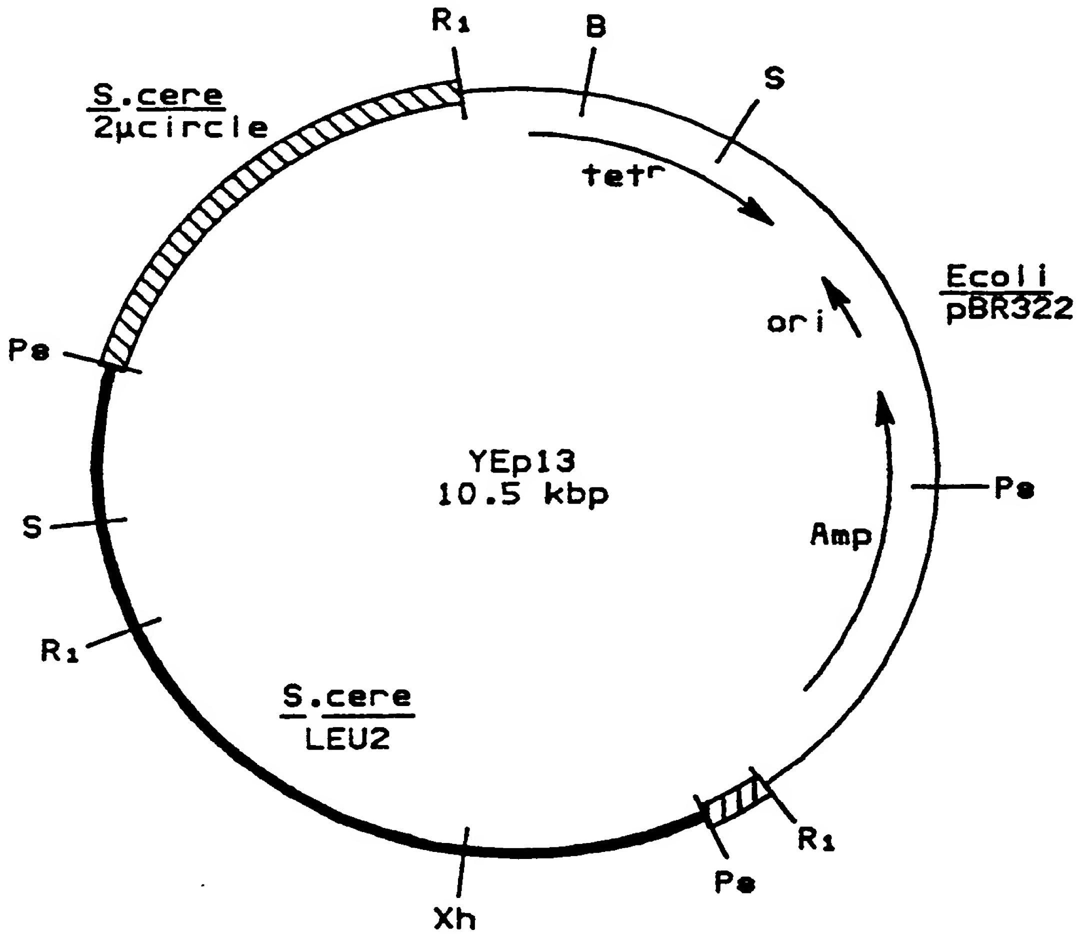
The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: NRRL

Y-15851, 15859

B-15874, 15889, 15892



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Ecoli
pBR322
ori
Ps
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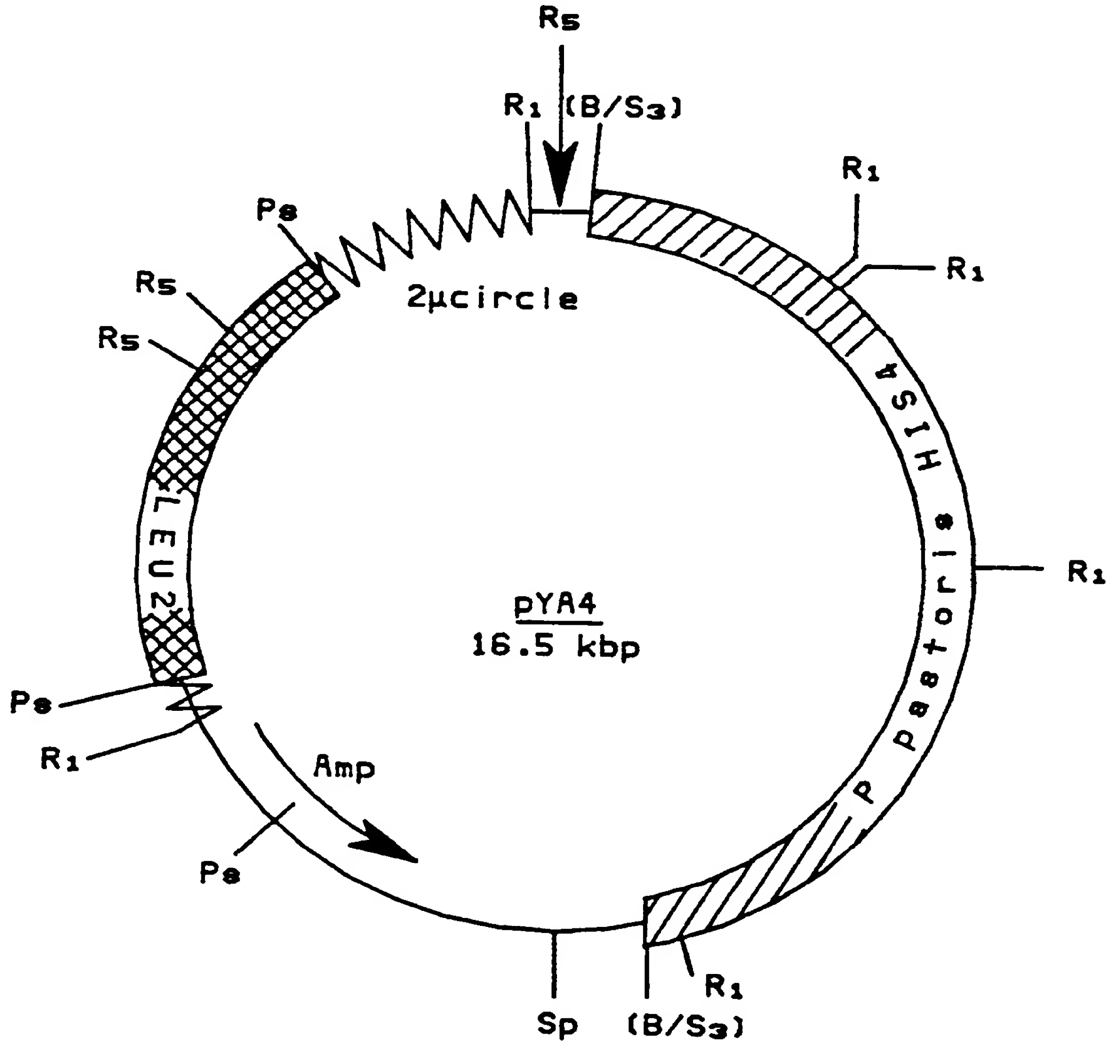
FIG. 1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R1
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Ps
R1
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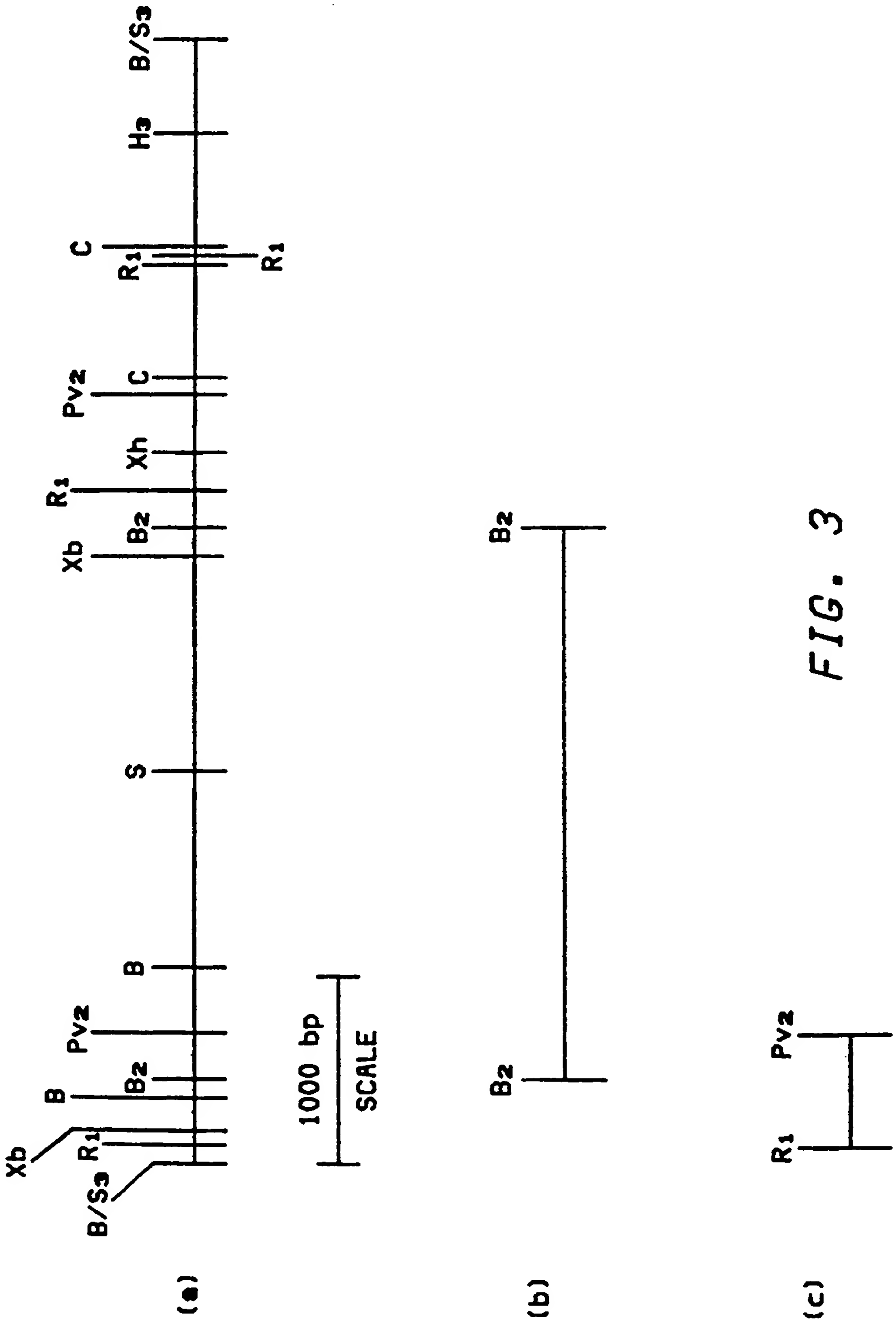
FIG. 2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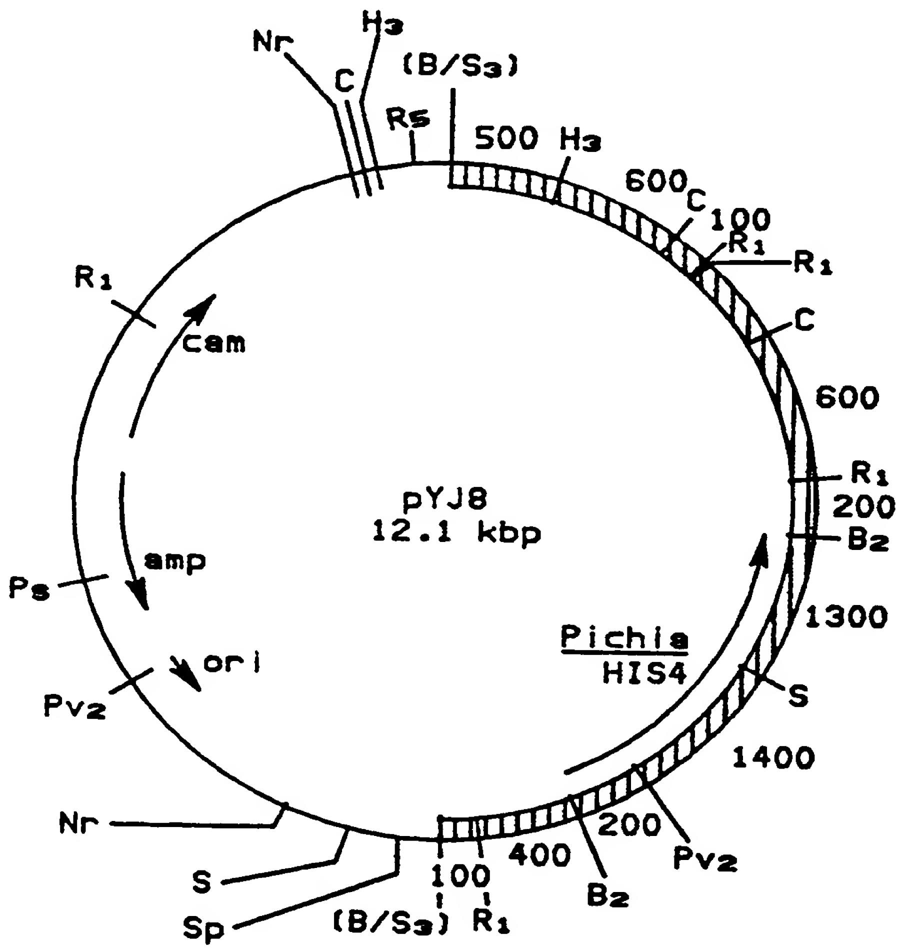
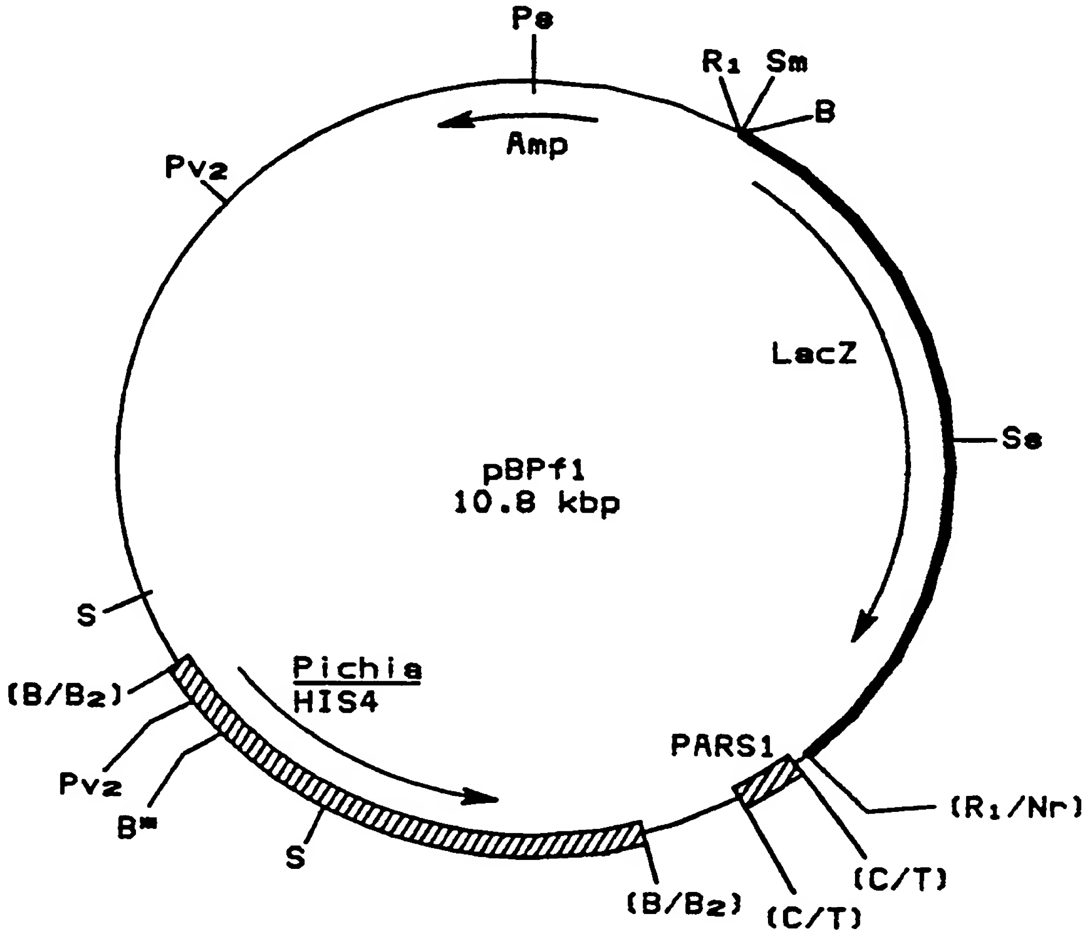
Xb
R1
B
B2
Pv2
B
S
Xb
B2
R1
Xh
Pv2
C
R1
C
R1
H3
B/S3
1000 bp
SCALE
(b)
B2
B2
(c)
R1
Pv2

FIG. 3



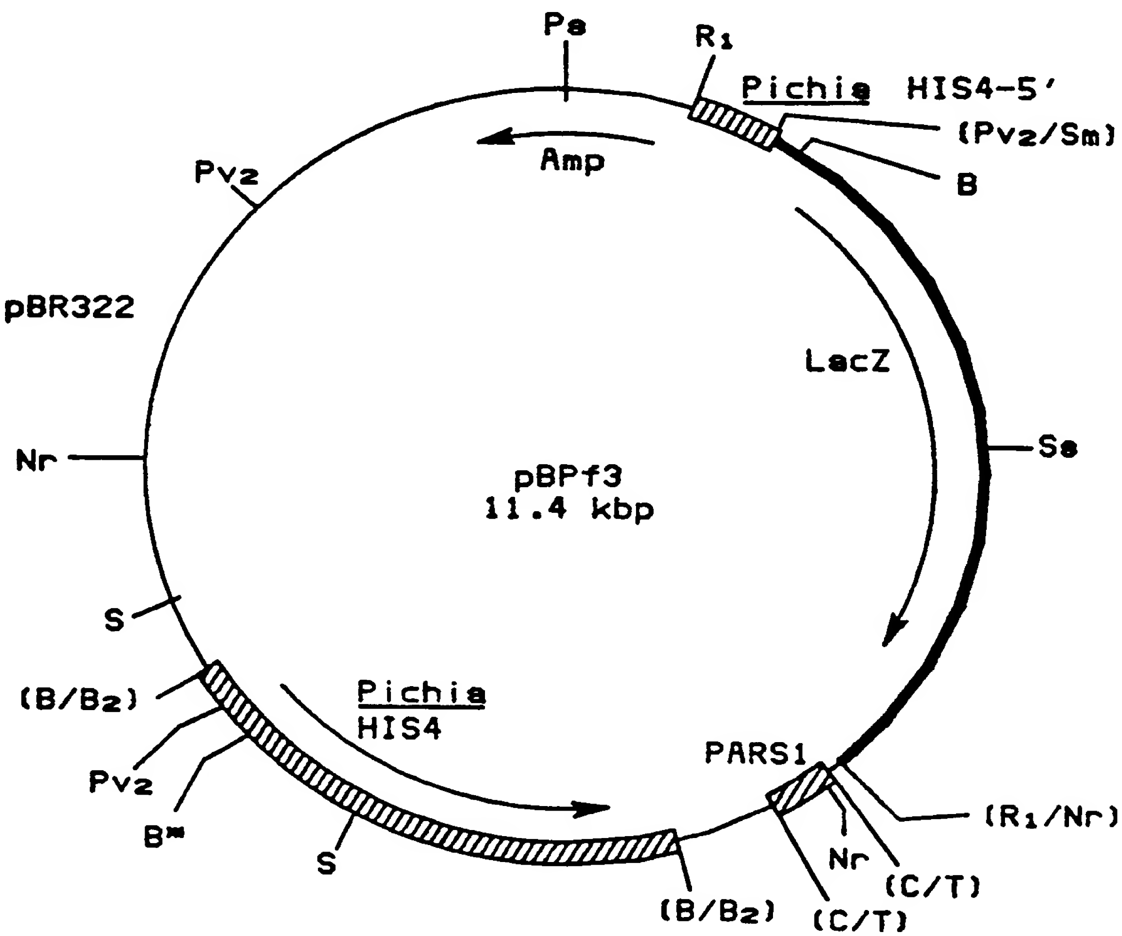
Nr
H3
C
(B/S3)
R5
500 H3
600
C
100
R1
R1
C
600
R1
200
B2
1300
S
1400
200
Pv2
B2
400
100
R1
(B/S3)
Sp
S
Nr
Pv2
ori
Ps
amp
R1
cam
pYJ8
12.1 kbp
Pichia
HIS4

FIG. 4



Ps
R1
Sm
B
Amp
Pv2
LacZ
Ss
pBPf1
10.8 kbp
S
Pichia
HIS4
(B/B2)
Pv2
B*
S
PARS1
(R1/Nr)
(C/T)
(B/B2)
(C/T)

FIG. 5



Ps
R1
Pichia HIS4-5'
(Pv2/Sm)
B
Amp
Pv2
pBR322
LacZ
Ss
Nr
pBPf3
11.4 kbp
S
(B/B2)
Pichia
HIS4
Pv2
B*
S
PARS1
(R1/Nr)
Nr
(C/T)
(B/B2)
(C/T)

FIG. 6